Phytochemistry, Vol. 28, No. 1, pp. 57–61, 1989.
Printed in Great Britain.

0031–9422/89 $3.00+0.00

ENZYMES OF CARBOHYDRATE METABOLISM IN SOYBEAN NODULES

LES COPELAND, JOHN VELLA and ZHENQUAN HONG

Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006, Australia

(*Received in revised form* 21 *June* 1988)

Key Word Index—*Glycine max*; Leguminosae; soybean; carbohydrate metabolism; enzymes; glycolysis; root nodules.

Abstract—The activities of enzymes of carbohydrate metabolism have been measured in the plant and bacteroid fractions of soybean (*Glycine max*) nodules and in roots of non-nodulated soybeans. The plant fraction of the nodules contained the enzymes required to convert sucrose to pyruvate and to oxaloacetate as well as enzymes of starch synthesis and degradation. In contrast, the bacteroids had only limited capacity for carbohydrate metabolism and appeared to lack the complete glycolytic pathway. The distribution of enzymes of carbohydrate metabolism between the cortical tissue and central infected zone of the nodules was investigated. The conversion of sucrose to organic acids may take place to a large extent in the cortical tissue of the nodules.

INTRODUCTION

Symbiotic nitrogen fixation in leguminous root nodules is dependent on the supply of carbohydrates from the host plant. The main carbohydrate translocated into nodules is sucrose [1], which is metabolized to provide nutrients for the bacteroids, energy and reductants for the nitrogenase reaction and carbon skeletons for the assimilation of fixed ammonia. Earlier investigations have shown that sucrose is cleaved in the plant fraction of soybean nodules by either alkaline invertase (EC 3.2.1.26) or sucrose synthase (EC 2.4.1.13) [2, 3]. The glucose and fructose produced in these reactions are likely to be further metabolized in the plant fraction of the nodules, following phosphorylation by a hexokinase (EC 2.7.1.1) and a fructokinase (EC 2.7.1.4) respectively [4]. There have been several reports of enzymes which may be involved in subsequent steps in the utilization of sucrose. For example, UDP-glucose pyrophosphorylase and several of the enzymes of the glycolytic and pentose phosphate pathways have been detected in the plant fraction of soybean nodules [5–8], and the plant cytosolic pyruvate kinase and phosphoenolpyruvate (PEP) carboxylase have been purified [9, 10]. Nevertheless, information on this aspect of root nodule metabolism is very limited. In the present study, we have measured the activities of all the enzymes of glycolysis, as well as selected enzymes of the pentose phosphate pathway and starch synthesis and breakdown in the plant and bacteroid fractions of soybean (*Glycine max* L. Merr., cv. Williams) nodules. The activities were also determined in roots of non-nodulated soybeans for comparison. The distribution of the enzymes between the cortical tissue and the central infected zone of the nodules was investigated.

RESULTS

Soluble extracts from the plant fraction of soybean nodules contained all of the enzymes required to convert sucrose to pyruvate and to oxaloacetate via the glycolytic pathway. The specific activities, determined under conditions that were optimized with respect to pH and substrate concentration, are shown in Table 1. The pentose phosphate pathway enzymes, glucose 6-phosphate dehydrogenase (Glc-6-P DH) and 6-phosphogluconate (6-PG) DH, were also present in the plant fraction of the nodules, as were enzymes of starch synthesis and breakdown (Table 1). The enzyme 3-hydroxybutyrate (3-HB) DH, which is a marker for bacteroids [11], was not detected in these extracts. Most of the enzymes surveyed had higher specific activities in the plant fraction of the nodules than in roots of non-nodulated soybeans (Table 1). UDP-Glucose phosphorylase activity [12] was not detected in nodule or root extracts (not shown).

To determine which of the enzymes were present in the bacteroids, activity was measured in bacteroid preparations before and after sonication. An increase in activity after sonication indicated that an enzyme was inside the bacteroids and not a contaminant from the plant fraction. Thus, 3-HB DH was detected only in sonicated bacteroid preparations (Table 2). Substantial increases in the activity of hexokinase, UDP-glucose pyrophosphorylase, phosphoglucomutase, phosphohexose isomerase, NAD-P-dependent Glc-6-P DH, NAD-dependent 6-PG DH and enzymes involved in the conversion of triose phosphates to pyruvate occurred following sonication, indicating that these enzymes were present inside the bacteroids (Table 2). Small increases were observed in the activities of NAD-dependent Glc-6-P DH and NADP-dependent 6-PG DH after sonication. However, there was no increase following sonication in the activities of alkaline invertase, sucrose synthase (assayed in the cleavage or synthesis directions), fructokinase, phosphofructokinase (PFK), phosphofructophosphotransferase (PFP), fructose 1,6-bisphosphate (Fru-1, 6-bisP) aldolase and PEP carboxylase, indicating that these enzymes were absent from the bacteroids (Table 2). ADP-glucose pyrophosphorylase, phosphorylase and amylase activities were not detected in the bacteroids before or after sonic-

ation (not shown). The activities of the enzymes in the bacteroids were much lower than those in the plant cytosol, when compared per g fr. wt nodules (Tables 1 and 2).

Nodule slices were digested with hydrolytic enzymes to prepare extracts from the cortical and central tissues. Leghemoglobin, 3-HB DH and uricase were present in extracts obtained from the central zone of the nodules but not the cortical tissue (Table 3). In contrast, alkaline invertase, sucrose synthase and fructokinase were present in the cortical tissue but were not detected in extracts of the central zone (Table 3). UDP-Glucose pyrophosphorylase, phosphoglucomutase, PFK, triose phosphate isomerase, Glc-6-P DH and 6-PG DH, as well as the enzymes of starch metabolism, also had substantially higher activity in extracts of the cortical tissue than the central zone (Table 3). The specific activities of phosphohexose isomerase, Fru-1,6-bisP aldolase, glyceraldehyde 3-phosphate DH, phosphoglycerate mutase and PEP carboxylase in the cortical tissue extracts were *ca* double those in extracts of the central zone (Table 3).

Table 1. Specific activities of enzymes of carbohydrate metabolism in the plant fraction of soybean nodules and roots of non-nodulated soybeans

Enzyme	nmol product formed/min. mg protein*	
	Nodules	Roots
Alkaline invertase†	111	34
Sucrose synthase	96	36
Hexokinase	18	18
Fructokinase	59	61
UDP-Glucose pyrophosphorylase	2510	1030
Phosphoglucomutase	439	211
Phosphohexose isomerase	338	188
PFK	95	50
PFP	35	45
Fru-1,6-bisP aldolase	25	27
Triose phosphate isomerase	9640	6820
Glyceraldehyde 3-phosphate DH	404	157
Phosphoglycerate kinase	2040	595
Phosphoglycerate mutase	746	461
Enolase	486	218
Pyruvate kinase	39	19
PEP carboxylase	153	18
Glc-6-P DH	159	177‡
6-PG DH	122	220‡
ADP-Glucose pyrophosphorylase	22	ND§
Starch phosphorylase	10	ND
α,β-Amylase	1580	116

*Except where indicated otherwise, the values are the means of at least three replicate extractions. The protein content of the extracts was 28±1 and 4.4±0.3 mg/g fr. wt nodules and roots respectively.

†Alkaline invertase, sucrose synthase, hexokinase and fructokinase activities from [2] have been included for comparison.

‡Mean of 2 extractions.

§Not detected.

Table 2. Enzymes of carbohydrate metabolism in bacteroids of soybean nodules

Enzyme	nmol product formed/min. g fr. wt*	
	Before sonication	After sonication
3-HB DH	ND†	1650±90
Alkaline invertase	ND	ND
Sucrose synthase	5±2	5±2
Hexokinase	37±30	198±40
Fructokinase	ND	ND
UDP-Glucose pyrophosphorylase	370±200	1830±360
Phosphoglucomutase	17±17	551±36
Phosphohexose isomerase	111±12	368±42
PFK	28±4	21±5
PFP	19±1	8±2
Fru-1,6-bisP aldolase	9±4	10±3
Triose phosphate isomerase	8790±2500	38 400±3300
Glyceraldehyde 3-phosphate DH	87±44	3160±220
Phosphoglycerate kinase	358±56	5500±380
Phosphoglycerate mutase	160±12	1450±230
Enolase	16±12	2080±140
Pyruvate kinase	21±16	613±35
PEP carboxylase	ND	ND
Glc-6-P DH (NADP)	12±12	132±31
Glc-6-P DH (NAD)	ND	15±7
6-PG DH (NADP)	ND	19±10
6-PG DH (NAD)	ND	121±6

*The values are the means±s.e.m of 3 replicate extractions.

†Not detected.

DISCUSSION

Bacteroids isolated from soybean nodules formed with *Bradyrhizobium japonicum* CB 1809 had limited capacity to utilize carbohydrates. No evidence was found in this study to indicate that enzymes of sucrose cleavage were in the bacteroids. Sucrose synthase, which is one of the major soluble proteins of soybean nodules [3], was present to a small extent in an unwashed bacteroid preparation in a previous study [2]. It now appears that this activity was the result of contamination from the plant enzyme. Traces of sucrose synthase activity have been reported in association with *B. japonicum* 61A76 bacteroids [8].

The absence of PFK, PFP and Fru-1,6-bisP aldolase indicated that *B. japonicum* CB 1809 bacteroids lacked a complete glycolytic pathway. A form of the pentose phosphate pathway, involving an NADP-dependent Glc-6-P DH and an NAD-dependent 6-PG DH, may operate in the bacteroids, but in view of the low activities of these enzymes such a pathway is unlikely to play a quantitatively important role in the production of energy. Bacteroids isolated from soybean nodules formed with other strains of *B. japonicum* have been reported to contain small amounts of PFK, Fru-1,6-bisP aldolase, NADP-dependent Glc-6-P DH and NAD-dependent 6-PG DH, but

Table 3. Activity of enzymes in extracts of the cortical tissue and central zone of soybean nodules

Enzyme	nmol product/min. mg protein*	
	Cortical tissue	Central tissue
Leghemoglobin†	ND‡	10.6±0.6
3-HB DH	3±1	99±8
Uricase	ND	43±3
Alkaline invertase	95±7	ND
Sucrose synthase	20±2	ND
Hexokinase	16±2	14±2
Fructokinase	47±6	ND
UDP-Glucose pyrophosphorylase	567±65	112±14
Phosphoglucomutase	369±12	96±6
Phosphohexose isomerase	428±1	255±63
PFK	92±5	17±2
PFP	32±2	17±1
Triose phosphate isomerase	4510±130	878±38
Glyceraldehyde-3-phosphate DH	160±13	81±9
Phosphoglycerate mutase	65±6	38±4
Enolase	32±4	26±1
PEP carboxylase	13±1	6±1
Glc-6-P DH	254±6	9±1
6-PG DH	161±3	8±1
ADP-Glucose pyrophosphorylase	16±1	ND
Starch phosphorylase	17±1	4±2
α-,β-Amylase	1460±90	ND

*The values are the mean±sem of 3 replicate experiments. The protein content of the cortical and central zone extracts was 2.1±0.2 and 1.0±0.1 mg/g fr. wt of nodules respectively.

†nmol/mg protein

‡Not detected

the activities were considered too low to support nitrogen fixation [5, 8].

Most of the capacity for carbohydrate utilization was in the plant fraction of soybean nodules. This finding is consistent with the generally held view that organic acids, rather than sugars, are the main substrates taken up by the bacteroids from the plant cytosol of the nodules [13, 14]. It is interesting to note that the specific activities of many of the enzymes involved in the conversion of sucrose to organic acids via the glycolytic pathway were increased by a factor of two or more in the plant fraction of the nodules compared to the roots. This suggests that synthesis of enzymes in the pathway of sucrose breakdown may be enhanced in the nodules, either through the expression of enzyme forms unique to nodules (i.e. nodulins) or increased synthesis of enzyme forms normally present in roots or other parts of the plant. A nodule specific form of sucrose synthase has been shown to occur in soybeans [15].

Both PFK and PFP were in the plant fraction of the nodules and in roots. The specific activities of PFK and PFP (assayed in the presence of the activator, Fru-2, 6-bisP) in extracts of roots were similar, but in the plant fraction of the nodules the catalytic potential of PFK was 2.7 times greater than that of PFP. This suggests that PFK plays a more important role than PFP in the pathway of sucrose utilization in soybean nodules. Salminen and Streeter [8] also reported much higher activity of PFK activity than PFP in the plant fraction of soybean nodules.

The cortical tissue of soybean nodules contains only uninfected cells, whereas the central zone contains many large infected cells, together with smaller uninfected interstitial cells [16, 17]. Information on the distribution of enzymes in the pathway of sucrose breakdown between the cortical and central tissues of the nodules was obtained using extracts prepared by macerating nodule slices with hydrolytic enzymes. A mixture of infected and uninfected cells was released from the central nodule tissue by the enzymic digestion, as shown by the presence of the markers leghemoglobin, uricase and 3-HB DH. Leghemoglobin occurs in the plant cytosol of infected cells [13], whereas uricase is predominantly in uninfected, interstitial cells in the central zone of soybean nodules [18]. Cortical tissue, which was free of infected and uninfected interstitial cells remained in the debris after enzymic digestion, as shown by the absence of leghemoglobin, 3-HB DH and uricase. The infected and uninfected cells isolated from the central zone of the nodules were not separated in this study. Methods involving density gradient centrifugation or filtration through nylon screens of different mesh size have been described for the separation of infected and uninfected cells from root nodules [19–21]. However, these methods were not found to give a clean separation of the two types of cells. Furthermore, as these methods select infected cells of a certain size only, it could be argued that the preparations obtained are not truly representative of the population of infected cells in the nodules.

Infected and uninfected interstitial cells in the central zone of root nodules appear to have different roles in the assimilation of the products of nitrogen fixation [e.g. see 22]. The localization of a nodule-specific uricase in the uninfected interstitial cells of soybean nodules [18] indicates that there may be metabolic differences between these cells and cells in the cortical tissue. The present investigation suggests that differences may also occur within soybean nodules with respect to the cellular location of enzymes of carbohydrate metabolism. Essentially all of the sucrose cleavage activity, together with the specific fructokinase, appeared to be in the cortical tissue. Enzymes which catalyse subsequent steps in the pathway of sucrose breakdown were also enriched in the cortical tissue, compared with the central zone of the nodules. This may mean that at least the initial steps in the pathway of sucrose utilization take place in the cortical tissue of soybean nodules. Compounds such as triose phosphates or organic acids may then be translocated into the central infected zone to provide substrates for nitrogen fixation and carbon skeletons for the assimilation of fixed ammonia. Some of the activity of a number of enzymes in the extracts from the central tissue of the nodules was inside the bacteroids. Thus, the catalytic potential of hexokinase, UDP-glucose pyrophosphorylase, phosphoglucomutase, phosphohexose isomerase,

and enzymes involved in the conversion of triose phosphate to pyruvate in the plant fraction of cells in the central zone of the nodules may be lower than indicated by our data. Glc-6-P DH and 6-PG DH had very much higher specific activities in the cortical tissue than in the central zone. The significance of this is not known. Activity of the enzymes of starch metabolism was also much higher in the cortical tissue. Starch has been shown to be present in only the uninfected cells of mature soybean nodules [16].

EXPERIMENTAL

Materials. Soybeans (*Glycine max.* L. Merr., cv. Williams) were grown in Perlite in a glasshouse with average day and night temperatures of 25 and 17° respectively. Nodulated plants were obtained from seeds that were inoculated and supplied with N-free nutrient soln as described [2]. For non-nodulated soybeans, seeds were not inoculated and 20 mM NH_4NO_3 was added to the nutrient soln.

Preparation of extracts. Unless stated otherwise, all steps were carried out at 4°. Nodules (1 g) were excised 40 to 55 days after planting and homogenized with a mortar and pestle in 10 ml of 50 mM Tris–HCl, pH 8, 5 mM $MgCl_2$, 1 mM EDTA and 20 mM 2-mercaptoethanol (buffer A) or 50 mM HEPES–KOH, pH 7.5, 25% (v/v) glycerol and 10 mM $MgCl_2$ (buffer B). The homogenate was centrifuged at 200 g for 5 min, the pellet discarded, and the supt centrifuged at 4000 *g* for 10 min. The 4000 *g* pellet was washed twice and resuspended in 5 ml of the respective homogenizing buffer. Half of the suspension was sonicated for 60 sec, centrifuged at 25 000 *g* for 10 min and the supt used for assays of sonicated bacteroid preparations. The remainder of the resuspended 4000 *g* pellet was used directly for assays of the unsonicated bacteroids. The 4000 *g* supt was centrifuged at 25 000 *g* for 10 min and the supt used for assays of enzymes in the plant fraction of the nodules. Roots (10 g) from non-nodulated soybeans were harvested 40 to 50 days after planting and blended for 60 sec in 30 ml of buffer A or buffer B. The homogenate was filtered through Miracloth, centrifuged at 25 000 g for 10 min and the supt used for the assay of enzymes in roots of non-nodulated soybeans. Buffer A gave the better recovery for all enzymes except phosphoglycerate kinase, pyruvate kinase and PEP carboxylase; the data for these enzymes are from extracts prepared in buffer B.

For the preparation of extracts from the cortical and the central infected tissues, nodules (3 g) were harvested 45–55 days after planting and cut into 1 mm slices in 20 mM MES–KOH, pH 5.8, 0.65 M D-mannitol, 2 mM $MgCl_2$, 4 mM $CaCl_2$ and 50 mM KCl (buffer C). The slices were rinsed (× 3) with fresh buffer C and incubated at 30° in 15 ml of buffer C to which 500 U cellulysin (Calbiochem–Behring), 20 U hemicellulase (Sigma) and 600 U pectinase soln (Sigma) had been added. The digestion mixture was shaken at *ca* 180 rotations/min for 1 hr and then at *ca* 60 rotation/min for 3 hr. The incubated mixture was filtered through 170 μm nylon mesh and the debris, retained by the mesh, washed twice with 10 ml buffer C. The combined filtrate and washings were centrifuged at 50 g for 4 min and the pellet washed × 4 by gently resuspending in 40 ml buffer C and spinning at 50 g for 4 min. The final washed pellet was resuspended in 7 ml of 50 mM HEPES–KOH, pH 7.5, 2 mM $MgCl_2$, 5 mM DTT, 0.4 mM EDTA (buffer D) to which insoluble PVP (0.3 g) was added. The suspension was sonicated for 60 sec, centrifuged at 20 000 g for 20 min and the supt used to assay enzymes from the central zone of the nodules. The nodule debris were pressed gently with a glass rod, washed × 5 with 15 ml buffer C and homogenized with a mortar and pestle in 15 ml of buffer D to which insoluble PVP (1.2 g) had been added. The homogenate was sonicated for 60 sec, centrifuged at 20 000 *g* for 20 min and the supt used for assays of enzymes from the cortical tissue.

Assay of enzymes. All enzyme assays were carried out at 30°, in reaction mixtures which had a final vol. of 1 ml. Extracts were dialysed for 2 hr against 100 vol. of the respective homogenizing buffer prior to the assay of all enzymes except Fru-1,6-bisP aldolase, pyruvate kinase, PEP carboxylase and 3-HB DH, which were assayed as soon as possible after extracts were prepared. Blank reaction mixtures without substrate were used to correct for non-specific reactions.

Conditions for the assay of the enzymes were optimized with respect to pH and the concentrations of all substrates, using the extract from the plant fraction of whole nodules. The composition of the optimized reaction mixtures are given. Amylase (α-amylase, EC 3.2.1.1 and/or β-amylase, EC 3.2.1.2): 50 mM NaOAc, pH 4.8, 5 mM $CaCl_2$ and 10 mg soluble starch; the reducing sugar released was estimated as described [23]. Phosphorylase (EC 2.4.1.1): 50 mM MES–KOH, pH 6.5, 50 mM Pi, 0.4 mg BSA and 10 mg soluble starch. UDP-glucose pyrophosphorylase (EC 2.7.7.9): 30 mM Tris–HCl, pH 8.5, 9 mM $MgCl_2$, 4 mM UDP-glucose, 5 mM PPi and 0.4 mg bovine serum albumin. ADP–glucose pyrophosphorylase (EC 2.7.7.12): 30 mM Tris–HCl, pH 8, 6 mM $MgCl_2$, 9 mM 3-phosphoglycerate (or 6 mM of Fru-1,6-bisP or pyruvate for bacteroid extracts), 1 mM ADP-glucose, 2 mM PPi and 0.4 mg BSA. The Glc-1-P formed in the preceding 3 assays was determined as described [24]. UDP-Glucose phosphorylase: assays were carried out in the pH range 6.5–9, in reaction mixtures as described for UDP-glucose pyrophosphorylase, except with PPi replaced by Pi and 20 μM Fru-2,6-bisP added. Alkaline invertase [2], sucrose synthase [3], hexokinase and fructokinase [4] were assayed as described.

The remaining enzymes were assayed by monitoring the change in *A* at 340 nm in continuous assays in which activity was coupled to the reduction of $NAD(P)^+$ or oxidation of NADH. All reactions were initiated by the addition of substrate and were linear for at least 3 min. Phosphoglucomutase (EC 2.7.5.1): 50 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$, 2.5 mM Glc-1-P, 75 μM Glc-1,6-bisP, 2 U Glc-6-P DH and 2 U 6-PG DH. Phosphohexose isomerase (EC 5.3.1.9): as for phosphoglucomutase, except that Glc-1-P was replaced by 2.5 mM Fru-6-P and Glc-1,6-bisP omitted. PFK (EC 2.7.1.11): as in [25] except that 30 mM Tris–HCl, pH 8, 6 mM $MgCl_2$, 5 mM Fru-6-P and 2.5 mM ATP were used and DTE omitted. PFP (EC 2.7.1.90): as for PFK except that ATP was replaced by 2.5 mM PPi and 20 μM Fru-2,6-bisP included. Fru-1,6-bisP aldolase (EC 4.1.2.13): 50 mM HEPES–KOH, pH 7, 1 mM Fru-1,6-bisP, 0.2 mM NADH, 2 U triosephosphate isomerase and 2 U α-glycerol-P DH. Triose phosphate isomerase (EC 5.3.1.1): 50 mM HEPES–KOH, pH 7, 5 mM glyceraldehyde-3-phosphate, 0.2 mM NADH and 2U α-glycerol-3-P DH. Glyceraldehyde-3-phosphate DH (EC 1.2.1.12): as in [26], except that 50 mM HEPES–KOH, pH 7, was used. Phosphoglycerate kinase (EC 2.7.2.3): as in [27], except that 50 mM HEPES–KOH, pH 7, 1.5 mM $MgCl_2$ and 1 mM ATP were used. Phosphoglycerate mutase (EC 2.7.3.5), as in method I of [28], except that 50 mM Tris–HCl, pH 8.5, 5 mM 3-phosphoglycerate and 0.6 mM 2,3-diphosphoglycerate were used and AMP and adenylate kinase omitted. Enolase (EC 4.2.1.11): as in [29], except that 50 mM Tris–HCl, pH 8, 8 mM $MgCl_2$ and 5 mM 2-phosphoglycerate were used. Pyruvate kinase (EC 2.7.1.40): as in [26], except that 50 mM HEPES–KOH, pH 7, and 2 mM PEP were used; in root extracts activity was also measured in reaction mixtures which contained 5 mM $NADP^+$, 5 mM glucose, 3U

hexokinase, 4U Glc-6-P DH and 0.3U 6-PG DH instead of NADH and lactate DH. PEP carboxylase (EC 4.1.1.31): as in [30], except that 50 mM HEPES–KOH, pH 7.5, was used. Glc-6-P DH (EC 1.1.1.49): 50 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$ or 1 mM NAD^+, 2.5 mM Glc-6-P and 0.3U 6-PG DH. 6-PG DH (EC 1.1.1.44): as for Glc-6-P DH except that 6-PG replaced Glc-6-P and 6-PG DH was omitted. Uricase (EC 1.7.3.3) [31], 3-HB DH (EC 1.1.1.30) [32], and leghemoglobin [33] were assayed as described. Protein was measured by the Lowry method [34] using bovine serum albumin as a standard.

Acknowledgements—This work was partially supported by the Australian Research Grants Scheme. J. V. was the recipient of an F. H. Loxton Scholarship of the University of Sydney. Nodulaid inoculum was a generous gift from Agricultural Laboratories, Sefton, N. S. W.

REFERENCES

1. Bach, M. K., Magee, W. E. and Burris, R. H. (1958) *Plant Physiol.* **33**, 118.
2. Morell, M. K. and Copeland, L. (1984) *Plant Physiol.* **74**, 1030.
3. Morell, M. K. and Copeland, L. (1985) *Plant Physiol.* **78**, 149.
4. Copeland, L. and Morell, M. K. (1985) *Plant Physiol.* **79**, 114.
5. Reibach, P. H. and Streeter, J. G. (1983) *Plant Physiol.* **72**, 634.
6. Tajima, S. and Yamamoto, Y. (1984) *Soil Sci. Plant Nutr.* **30**, 85.
7. Salminen, S. O. and Streeter, J. G. (1986) *Plant Physiol.* **81**, 538.
8. Salminen, S. O. and Streeter, J. G. (1987) *Plant Physiol.* **83**, 535.
9. Peterson, J. B. and Evans, H. J. (1978) *Biochim. Biophys. Acta* **567**, 445.
10. Peterson, J. B. and Evans, H. J. (1978) *Plant Physiol.* **61**, 909.
11. Brown, C. M. and Dilworth, M. J. (1975) *J. Gen. Microbiol.* **86**, 39.
12. Gibson, D. M. and Shine, W. E. (1983) *Proc. Natl Acad. Sci. U.S.A.* **80**, 2491.
13. Appleby, C. A. (1984) *Annu. Rev. Plant Physiol.* **35**, 443.
14. Dilworth, M. J. and Glenn, A. R. (1984) *Trends Biochem. Sci.* **9**, 519.
15. Thummler, F. and Verma, D.P.S. (1986) in *Molecular Genetics of Plant–Microbe Interactions* (Verma, D. P. S. and Brisson, N., eds), p. 382, Martinus Nijhoff, Dordrecht.
16. Bergersen, F. J. and Goodchild, D. A. (1973) *Aust. J. Biol. Sci.* **26**, 729.
17. Selker, J. M. L. and Newcomb, E. H. (1985) *Planta* **165**, 446.
18. Van den Bosch, K. A. and Newcomb, E. H. (1986) *Planta* **167**, 425.
19. Hanks, J. F. Tolbert, N. E. and Schubert, K. A. (1983) *Plant Physiol.* **68**, 65.
20. Shelp, B. J., Atkins, C. A. and Storer, P. J. (1983) *Arch. Biochem. Biophys.* **224**, 429.
21. Suganama, N., Kitou, M. and Yamamoto, Y. (1987) *Plant Cell Physiol.* **28**, 113.
22. Schubert, K. R. (1986) *Annu. Rev. Plant Physiol.* **37**, 539.
23. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
24. Bergmeyer, H. U. and Michal. G. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 1233. Academic Press, New York.
25. Kelly, G. J. and Latzko, E. (1977) *Plant Physiol.* **60**, 295.
26. Journet, E. P. and Douce, R. (1985) *Plant Physiol.* **79**, 458.
27. Scopes, R. K. (1975) *Methods Enzymol.* **42**, 127.
28. Stitt, M. and ap Rees, T. (1979) *Phytochemistry* **18**, 1905.
29. ap Rees, T., Thomas, S. M., Fuller, W. A. and Chapman, B. (1975) *Biochim. Biophys. Acta* **385**, 145.
30. Wong, K. F. and Davies, D. D. (1973) *Biochem. J.* **131**, 451.
31. Hanks, J. F., Tolbert, N. E. and Schubert, K. R. (1981) *Plant Physiol.* **68**, 65.
32. Wong, P. P. and Evans, H. J. (1971) *Plant Physiol.* **47**, 750.
33. Appleby, C. A. and Bergerson, F. J. (1980) in *Methods for Evaluating Biological Nitrogen Fixation* (Bergerson, F. J., ed.), p. 315. Wiley, Chichester.
34. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.